American Journal of Pathology, Vol. 136, No. 5, May 1990

A Unique Phenotype of Skin-associated Lymphocytes in Humans

Preferential Expression of the HECA-452 Epitope by Benign and Malignant T Cells at Cutaneous Sites

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It has been proposed that the skin is a functionally unique compartment of the immune system, although little direct evidence supporting this hypothesis has been presented. Here we show that lymphocyte populations at cutaneous sites can be differentiated from otherwise similar populations at noncutaneous sites by their preferential expression of an epitope defined by the MAb HECA-452. This MAb recognizes a predominantly 200-kd cell-surface glycoprotein present on about 16% of peripheral blood T cells, including both CD4+ and CD8+ T cells (17% and 11% HECA-452+, respectively), as well as TCR-δ–bearing T cells (32%+). Most thymocytes (99%) lacked HECA-452 antigen expression, and essentially all the HECA-452+ peripheral blood T cells were found in the adhesion molecule^{bigb}, CD45R^{low} putative memory cell subset, findings suggesting that HECA-452 expression develops peripherally as a consequence of antigenic stimulation. However, the HECA-452 antigen is not a conventional activation antigen because it was not upregulated with mitogen stimulation of peripheral blood T cells. Most significantly, among 54 diverse specimens of normal/reactive lymphoid tissues and sites of chronic inflammation, there was a clear association of lymphocyte HECA-452 expression and cutaneous location. In extracutaneous sites (n = 38) only about 5% of lymphocytes within the T-cell areas of these tissues expressed this antigen, whereas in inflammatory skin lesions (n = 16), 85% were HECA-452+. The association of HECA-452 expression and cutaneous location was also seen in a series of T-cell lymphomas. The malignant cells of 16 of 18 cases of epidermotropic (patch/plaque) stage mycosis fungoides were HECA-452+, as well as 2 of 7 nonmycosis fungoides peripheral T-cell lymphomas in skin. In contrast, this antigen was not expressed in thymic (lymphoblastic) lymphomas (n = 14), nonepidermotropic (tumor) stage mycosis fungoides (n = 5), and noncutaneous peripheral T-cell lymphomas (n = 15). Among lymphocytes, the preferential expression of the HECA-452 determinant by cutaneous T cells supports the hypothesis that the skin constitutes a immunologically unique lymphoid tissue and suggests that this molecule may play a role in either lymphocyte homing to skin or in lymphocyte interactions with the epidermis. (Am J Pathol 1990, 136:1053–1068)

The maintenance of immune homeostasis requires the precise coordination of functionally diverse lymphoid and accessory cell types in a wide variety of tissue environments. Several different lines of evidence suggest that components of this complex system are not evenly dispersed throughout the various lymphoid tissues, but instead are distributed in a regionally or anatomically restricted manner. An example of such regional specializa-

Supported in part by Grant AI19957 from the National Institute of Health, an award from the Veterans Administration, and by the Digestive Diseases Center FACS Core facility under DK38707.

Louis J. Picker and Sara A. Michie are Career Development Awardees of the Veterans Administration. Eugene C. Butcher is an Established Investigator of the American Heart Association.

Accepted for publication December 15, 1989.

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tion is the preferential localization of IgA-producing plasma cells, their precursors, and the T cells required for the maturation of these precursors in gut-associated lymphoid tissues (GALT) in close proximity to the gut lumen in which secretory IgA is destined to function.[1,2] The existence of a distinct compartmentalized GALT is further supported by the identification of cell-surface antigens in both humans and rats that are preferentially expressed on intestinal lymphocytes,[3,4] and by studies showing that certain gut-derived lymphoid populations are GALT specific in their homing/recirculation properties. Indeed, the best documented regional lymphocyte homing specificities involve GALT *versus* peripheral lymph node[5–10] systems in which the molecular basis of homing specificity has been demonstrated to reflect interactions between distinct adhesion molecules on the lymphocyte cell surface (homing receptors), and tissue-specific ligands (vascular addressins) expressed on the surface of the specialized lymphocyte-transmitting high endothelial venules (HEV[11]).

Another proposed candidate for regional immunologic specialization is the skin. Normal skin has been shown to have characteristic lymphoid components (the skin-associated lymphoid tissue [SALT]) in a microenvironment sufficient to allow specific recognition of and response to foreign antigens.[12–14] The enhanced ability of some lymphoid populations to migrate to cutaneous inflammatory lesions,[15–17] and the propensity for certain T-cell malignancies (mycosis fungoides and related disorders) to remain localized to skin for long periods of time[18–20] suggests that the skin may be a distinct regional lymphocyte homing specificity, but direct evidence of a phenotypically and/or functionally distinct compartmentalized SALT is lacking. Here we report that reactive T cells in cutaneous sites of inflammation, as well as the malignant T cells of mycosis fungoides, can be phenotypically differentiated from populations of benign or malignant T cells in noncutaneous sites by their expression of a novel cell-surface antigen defined by the MAb HECA-452. These data suggest the existence of a skin-specific lymphocyte phenotype and provide significant new evidence supporting the hypothesis that SALT is an immunologically unique lymphoid organ.

Materials and Methods

Tissues and Cell Preparation

Samples of snap-frozen human tissues for immunostaining were obtained from the Department of Pathology's frozen tissue bank at Stanford University, except for synovial tissue and salivary gland specimens, which were provided by Drs. S. Jalkanen (University of Turku, Finland) and N. Wu (Stanford University), respectively. Diagnostic classification of all specimens was done on the basis of standard pathologic, and as appropriate, clinical criteria. Tonsil lymphocytes and thymocytes were obtained by gentle mincing and washing of fresh, pathologically benign pediatric tonsils or whole thymic lobes over type 304 steel screen (Tylenter, Mentor, OH) in RPMI-1640 media (GIBCO, Grand Island, NY) with 2% fetal calf serum (FCS). The collected thymocytes were washed twice in the same media before use, whereas tonsil lymphocytes were further purified by centrifugation over Ficoll-Hypaque (Histopaque 1077; Sigma Chemical Co., St. Louis, MO), and then were washed twice before use.

Peripheral blood buffy coats from normal adult donors were separated into high-density (≥98% polymorphonuclear leukocytes [PMNs] by morphology) and low-density (peripheral blood mononuclear cells [PBMC]; 80% to 85% lymphocytes and 15% to 20% monocytes) populations by Ficoll-Hypaque two-step gradient density sedimentation (Histopaque 1077 and 1119; Sigma). The PBMC population was further separated into purified monocyte, lymphocyte (PBL), and T-cell populations, as previously described.[21] Briefly, PBMCs were adhered to plastic petri dishes (Falcon, Oxnard, CA) and separated into adherent (90% to 95% monocytes), and nonadherent (≥95% lymphocytes) populations. T-cell enriched populations were obtained by rosetting the nonadherent cells with 2-amino ethyl isothiouronium bromide hydrobromide (Sigma) -treated sheep erythrocytes. This latter population was 99% CD2+ when analyzed by flow cytometry (FACS analysis; below) using lymphocyte gates. The U937 human monocytoid cell line was originally obtained from the American Type Culture Collection (ATCC CRL 1593, Rockville, MD)

Activation of PBMC

Peripheral blood mononuclear cells were stimulated with phytohemagglutinin (PHA) at 1:100 final dilution of stock (GIBCO) or with 5 to 10 μg/ml Concanavalin A (Con A; Sigma) in RPMI-1640 media supplemented with 10% autologous serum. The cells were cultured at 1×10^6/ml for 3 to 14 days in a humidified incubator with 10% CO_2 at 37°C. In some experiments, cells from the same donor were incubated without mitogen under identical conditions. The culture media were periodically changed to maintain optimal conditions for cell growth.

MAbs

The production of the HECA-452 MAb (rat IgM) has been described previously.[22] The OZ-42 (against a mouse cere-

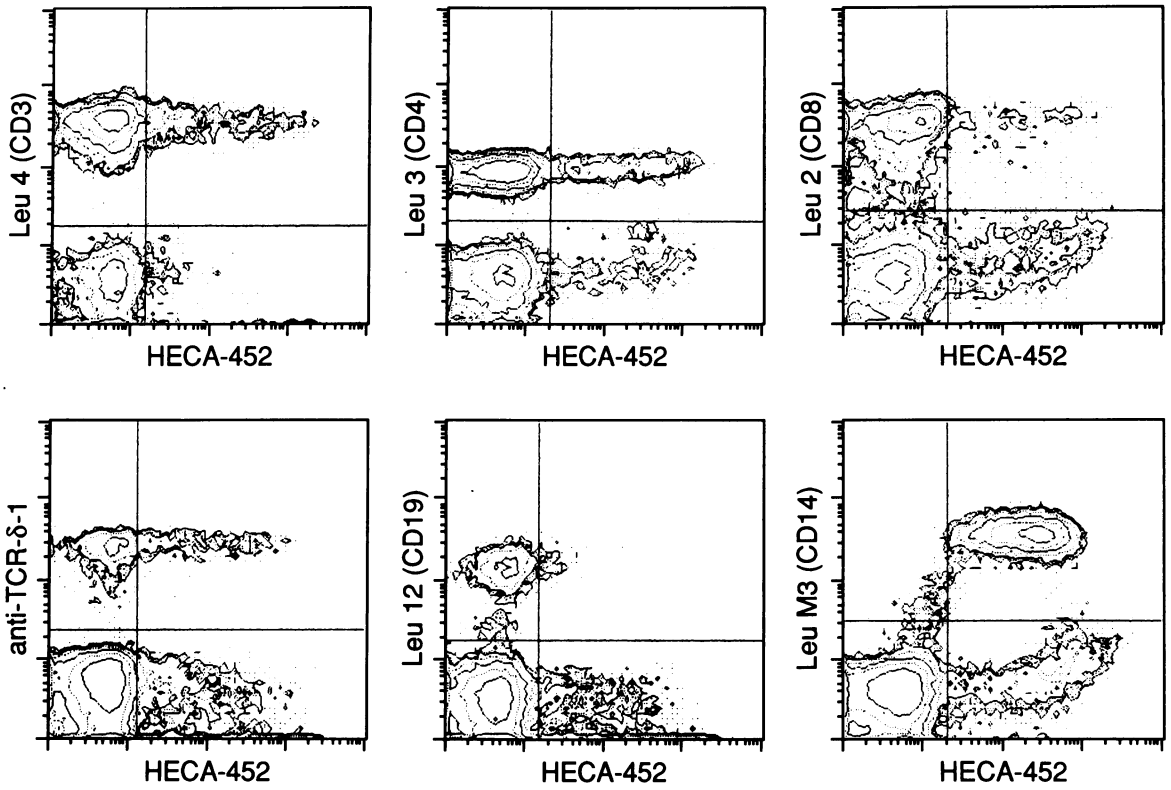

Figure 1. *The HECA-452 antigen defines a novel subset of peripheral blood T cells, is poorly expressed by peripheral blood B cells, and is uniformly expressed by monocytes. Representative contour plots (all plots are not from the same experiment) of two-color FACS analysis (log scale) of PBMC for HECA-452 (x axis)* versus *hematolymphoid differentiation markers (y axis). The Leu M3 (CD14) contour plot shows the whole PBMC population; all other plots are gated for lymphocytes. Quadrants were determined on the basis of appropriate negative controls (see text).*

bellar antigen[23]) and MECA-79 (against the mouse peripheral lymph node addressin[24]) MAbs were used as species- and isotype-matched controls. Purified preparations of these MAbs were conjugated to fluorescein isothiocyanate (FITC) according to the method of Goding.[25] The Leu 2 (CD8, MHC class I-restricted T subset), Leu 3 (CD4, MHC class II-restricted T subset), Leu 4 (CD3, T lineage), Leu 5 (CD2, T lineage), Leu 9 (CD7, T lineage), Leu M1 (CD15, myeloid), Leu M3 (CD14, monocyte/macrophage), Leu 18 (CD45R), and Leu 12 (CD19, B lineage) MAbs (both FITC-labeled and unconjugated) were obtained from Becton-Dickinson (Mountain View, CA). The anti-transferrin receptor MAb OKT9 (CD71) was obtained from Ortho Diagnostics (Raritan, NJ), and FITC-conjugated 4B4 MAb (CD29, VLA β-chain) from Coulter Immunology (Hialeah, FL). The anti-TCR-δ-1 MAb was a gift of Dr. M. Brenner (Boston, MA[26]). The anti-LFA-1 β chain MAb TS1/18 and the anti-LFA-3 MAb TS2/9 were provided by Drs. A. Krensky and C. Clayberger (Stanford, CA[27]). The anti-human H-CAM (CD44/Pgp-1) MAbs Hermes-3 and H2-7 were produced in our own laboratory.[28] All antibodies were used at saturating concentrations as determined by both FACS analysis and immunoperoxidase techniques.

Flow Cytometry

Cell populations (1×10^6 cells/test) were incubated with a primary unconjugated mouse MAb, washed twice in PBS, incubated with a Phycoerythrin (PE)-conjugated anti-mouse IgG (Tago, Burlingame, CA), washed with PBS, blocked (10 minutes) with 5% normal mouse serum (NMS)/5% normal rat serum (NRS) in PBS, and then incubated with FITC-HECA-452 or control MAb. All antibody incubation periods were for 30 minutes at 4°C in the presence of 0.2% sodium azide. After washing twice, the stained cells were either analyzed immediately or fixed in 1% paraformaldehyde in PBS and saved at 4°C for later analysis. In some experiments, unconjugated HECA-452 or control MAbs were used as initial antibodies followed by staining with PE-conjugated anti-rat IgG (Tago, heavy- and light-chain reactive), and then FITC-labeled mouse

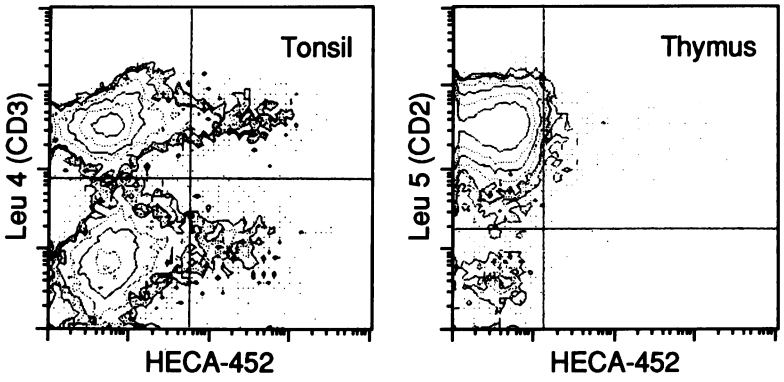

Figure 2. *A significant subset of tonsil T cells, but only rare thymocytes express the HECA-452 antigen. Two-color FACS analysis (log scale) of tonsil lymphocytes and thymocytes (whole populations) for HECA-452 (x axis)* versus *Leu 4 (CD3) and Leu 5 (CD2) MAbs (y axis), respectively. Quadrants were determined on the basis of appropriate negative controls (see text).*

MAbs. These two staining techniques gave identical results.

Flow cytometry analysis was performed on a FACStar (Becton Dickinson Immunocytometry Systems, Mountain View, CA) equipped with an argon laser that was operated at 488 nm. The FITC and PE emissions were collected with 530/30 and 585/42 band pass filters, respectively. All data was collected in list mode, ungated. For analysis, gates were drawn to include the whole population, excluding only doublets and larger cell aggregates, or were drawn around the lymphocyte, monocyte, and granulocyte regions as appropriate. Data analysis was done with Consort 30 software (Becton-Dickinson Immunocytometry Systems). The data are represented on contour plots

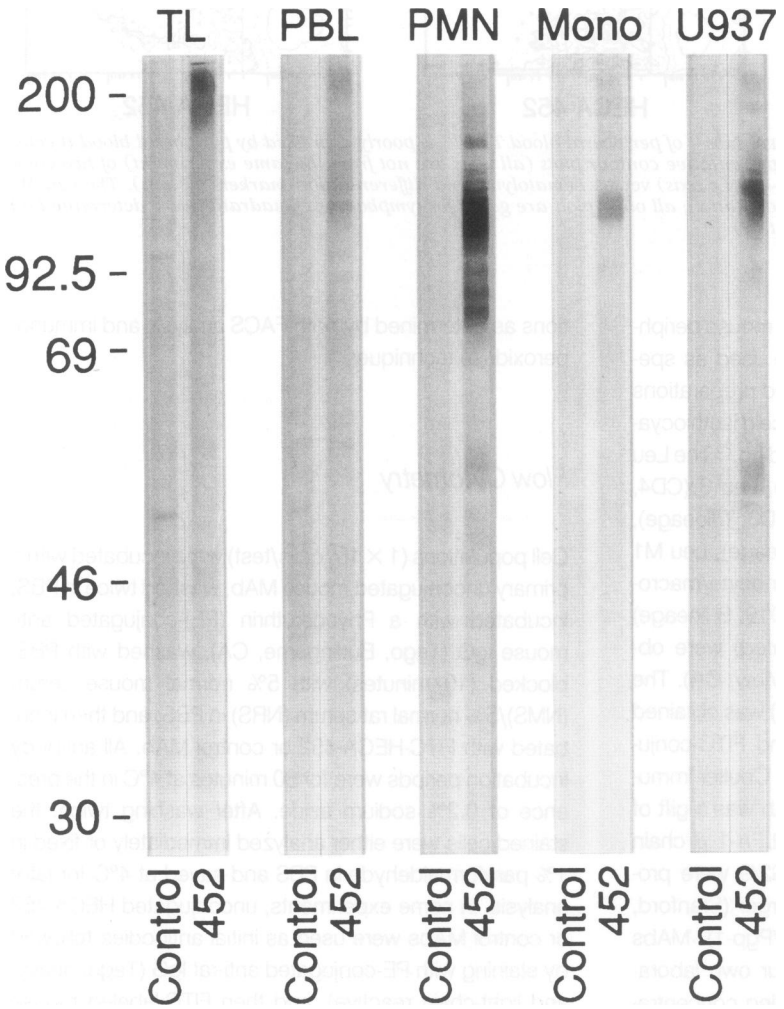

Figure 3. *The HECA-452 MAb recognizes different molecular species on different leukocyte subsets. Western blot of tonsil lymphocyte (TL), peripheral blood lymphocyte (PBL), polymorphonuclear leukocyte (PMN), monocyte (mono), and U937 monocytoid cell line lysates stained with the HECA-452 MAb* versus *an isotype-matched control (OZ-42). The relative position of molecular weight markers are indicated at left (×1000 kd).*

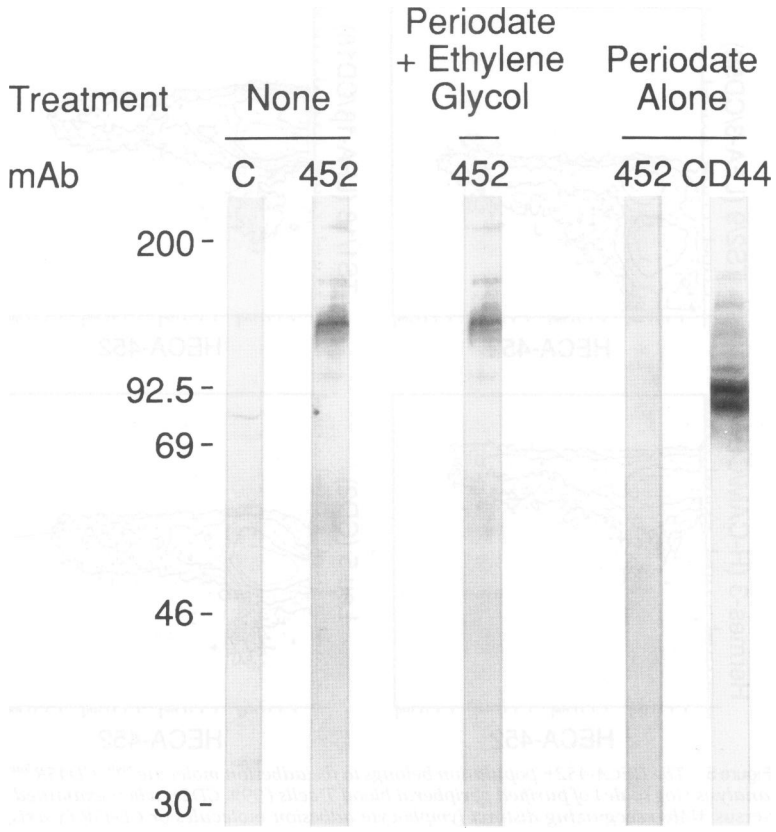

Figure 4. *The HECA-452 epitope is sensitive to periodate oxidation. Nitrocellulose strips with blotted U937 lysates were treated with periodate or periodate plus ethylene glycol (an inhibitor of periodate oxidation), and compared to untreated strips for reactivity with the HECA-452 MAb. Periodate treatment abrogated the ability of the HECA-452 MAb to recognize its antigen but showed no effect on the reactivity of control MAbs known to recognize carbohydrate-independent epitopes on the 90-kd HCAM (CD44) glycoprotein (see text). Staining with the isotype-matched-control MAb OZ-42 (C) is shown only for control-untreated blots. The relative position of molecular weight markers are indicated at left (×1000 kd).*

with the lowest level at 2 or 3 cells and the contours drawn at 2, 4, 8, 16, 32, 64, and 128 cells. The delineation and quantitation of positively stained populations (ie, placement of marker quadrants) was based on samples stained with isotype-matched antibody controls (positive quadrants with ≤0.7% cells in control plots).

Electrophoresis/Western Blotting

Lysates were prepared by incubating 2 to 4 × 10^7 cells in 1 ml lysis buffer (2% Nonidet P-40, 150 mmol/l [millimolar] NaCl, 1 mmol/l $MgCl_2$, 10 μg/ml Aprotinin, and 1 mmol/l phenyl methyl sulphonyl fluoride in 20 mmol/l Tris-HCl, pH 7.5) for 45 minutes at 4°C, followed by centrifugation at 10,000*g* for 30 minutes. Aliquots of these lysates were applied to 8% SDS-PAGE gels under reducing conditions.[29] Proteins were transferred to nitrocellulose with a Biorad (Richmond, CA) transblot apparatus. After blocking nonspecific protein binding with 100% horse serum for 30 minutes, primary and secondary antibody incubations (alkaline phosphatase conjugated anti-rat IgM [Zymed, So. San Francisco, CA] for HECA-452 and controls or anti-mouse IgG [Promega, Madison, WI] for HCAM/CD44 MAbs) were done in a Miniblotter 25 staining apparatus (Immunetics, Cambridge, MA; 1 hour incubation at room temperature for each). Washes between incubations were with TBST (10 mmol/l Tris-HCl, 150 mmol/l NaCl, 0.05% Tween-20, pH 8.0). The alkaline phosphatase reaction was developed as described by Promega. For periodate oxidation experiments, strips of nitrocellulose with blotted proteins were treated with 20 mmol/l sodium periodate in 50 mmol/l acetate buffer (pH 4.5) ± 250 mmol/l ethylene glycol for 40 minutes at room temperature in the dark. After washing with TBST, the treated nitrocellulose strips were stained as described above.

Tissue Section Immunostaining

Serial, acetone-fixed, air-dried cryostat sections (5 to 6 μm) were prepared from snap-frozen tissue specimens and stained using a 3-stage immunoperoxidase technique. Sections were incubated serially (45 minutes in a humidified chamber with PBS washes in between) with primary MAbs, biotinylated secondary antibodies—goat anti-rat IgM (Kirkegaard & Perry Labs, Gaithersburg, MD), or horse anti-mouse IgG (Vector Labs, Burlingame, CA)—as appropriate for the primary MAb, horseradish peroxidase-conjugated Streptavidin (Zymed), and then developed with 0.05% 3,3 diaminobenzidine (Sigma) and

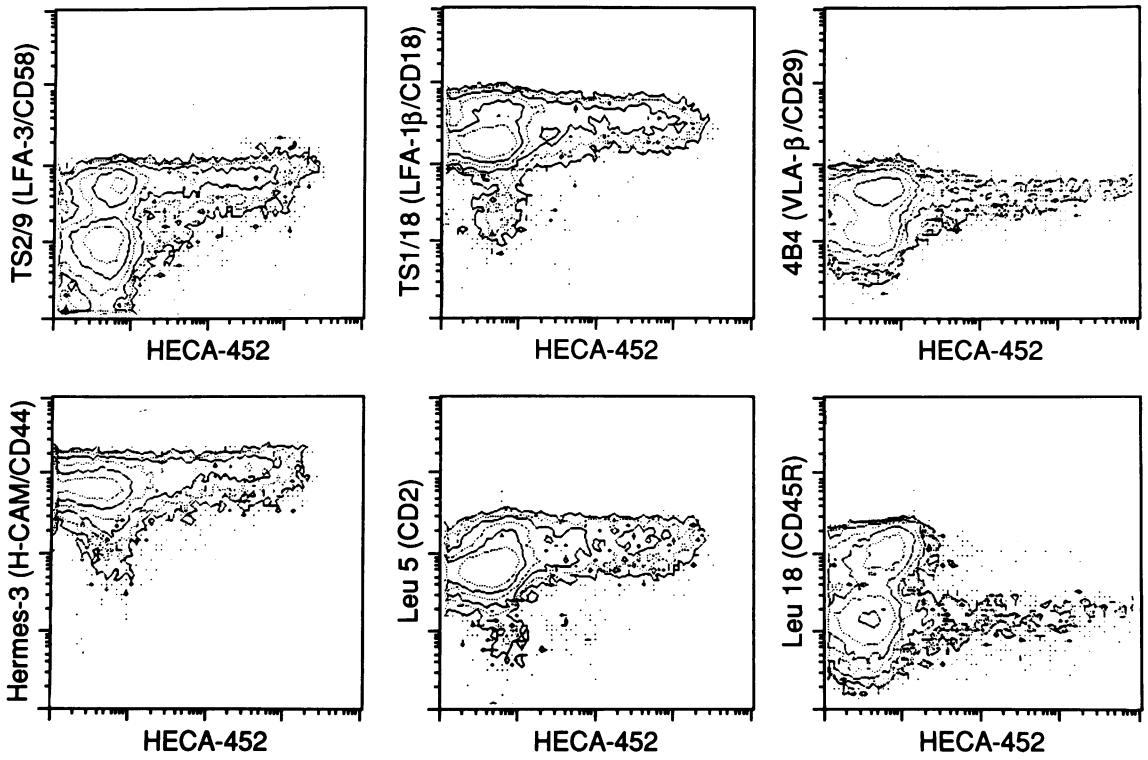

Figure 5. *The HECA-452+ population belongs to the adhesion molecule ^{bigb}, CD45R ^{low} putative memory T-cell subset. Two-color FACS analysis (log scale) of purified peripheral blood T cells (99% CD2+ when examined using lymphocyte gates) for HECA-452 (x axis)* versus *MAbs recognizing distinct lymphocyte adhesion molecules or CD45R (y axis). Contour plots for TS 2/9, TS 1/18, Hermes-3, and Leu 5 MAbs were from an experiment using the same T-cell preparation, whereas the 4B4 and Leu 18 plots were derived from an experiment using T cells from a different individual. Overall, identical results have been obtained using cells from four different blood donors.*

0.009% hydrogen peroxide in 50 mmol/l Tris/HCl (pH 7.5) for 10 minutes. Second and third stages included 5% normal human serum (NHS) to decrease background. After darkening the reaction with 0.5% copper sulfate in 0.9% NaCl for 5 minutes, sections were counterstained with 2% methylene blue, dehydrated, and coverslipped.

Two-color tissue section or cytospin immunofluorescence (HECA-452 *vs.* Leu 4 or other mouse MAbs) was accomplished using sequentially a 2-stage detection system for mouse IgG followed by an appropriate blocking step and then a 3-stage system for rat IgM. Briefly, sections were incubated (30 minutes in a humidified chamber for each incubation step) with a mouse IgG MAb followed by rhodamine-conjugated anti-mouse IgG (Sigma). After blocking 5 minutes with 5% NMS/5% NRS in PBS, the sections were incubated with the primary rat IgM MAb (ie, HECA-452 or control), followed by biotinylated anti-rat IgM (Kerkegaard and Perry Labs) in PBS with 2% NHS/5% NMS, and finally by FITC-conjugated avidin (Becton-Dickinson).

Immunohistologic Interpretation/Quantitation

The immunoarchitecture of all cases was defined with CD3 (T cell), CD19 (B cell), and CD14 (macrophage/monocyte) MAbs. In some cases, CD15 MAb was used to define tissue PMNs. Serial sections were then evaluated for HECA-452 *versus* control MAbs. The number of HECA-452+ cells with lymphoid morphology were evaluated in T-cell zones only, defined as areas containing at least 80% CD3+ cells; morphologically discernible PMNs, macrophages, endothelial cells, dendritic cells, fibroblasts, and epithelial cells were not considered. An average of 590 cells (range, 434 to 829 cells) were counted in at least five separate fields. In small specimens or those with focal infiltrates, multiple sections at different levels of the tissue block were evaluated. Two-color immunofluorescence analysis (CD3 *vs.* HECA-452, as described above) was used in 12 cases, including 5 cutaneous and 7 extracutaneous infiltrates, to check the accuracy of this evaluation, and in all instances similar results were obtained. The T-lineage lymphoma cases were evaluated differently. In these cases, the malignant cell population (which was usually intermixed with variable numbers of reactive cells) was determined by morphologic and immunophenotypic criteria,[30] and the malignant cells were evaluated for specific HECA-452 reactivity. A case was considered positive if 20% or more of the malignant population was clearly HECA-452+.

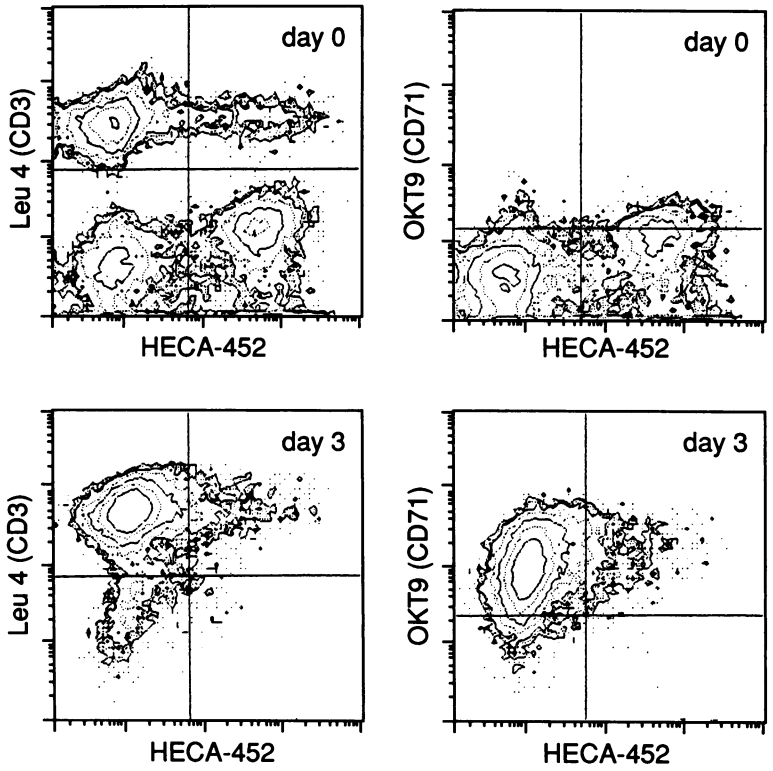

Figure 6. *T-cell HECA-452 antigen is not up regulated with mitogen stimulation. Representative two-color FACS analysis of PBMC for the T-cell marker CD3 and the activation antigen CD71 (transferrin receptor)* versus *HECA-452 before and after stimulation with the mitogen PHA. The plots shown are for the whole PBMC population; however, whereas monocytes are present in the day 0 plots, due to adherence to plastic during* in vitro *culture they are absent in the day-3 plots. Quadrants were determined on the basis of appropriate negative controls (see text).*

Results

A T-cell Subset Displays HECA-452 Reactivity

The HECA-452 MAb was originally described as a marker of high endothelial differentiation that also reacted with a poorly characterized monocytoid cell population in tissue sections.[22] Subsequent work showed that this MAb also reacted with PMNs and myeloid precursors in sections of bone marrow.[31] Two-color flow cytometry was used to more precisely define patterns of HECA-452 among populations of peripheral blood leukocytes. As shown in Figure 1, the HECA-452 epitope is expressed on a subpopulation of both CD3+ T cells and CD19+ B cells, and on essentially all CD14+ monocytes. Similar analysis of isolated PMN populations also revealed essentially 100% surface reactivity (not shown). In six different donors the mean percentage (and range) of HECA-452+ T cells was 16% (8% to 23%). About 11% (6% to 14%) of peripheral B cells were HECA-452+; however, the HECA-452 staining intensity of these cells was quite low (barely above background; Figure 1). Roughly similar subsets of both CD4+ (class 2 MHC-restricted), and CD8+ (class 1 MHC-restricted) T cells were HECA-452+, 17% (10% to 23%) and 11% (9% to 15%), respectively (p = NS, Students *t*-test). Interestingly, a somewhat larger subset of T-cell receptor-delta (TCR-δ) bearing T cells—32% (28% to 36%, three experiments)—expressed the HECA-452 determinant. HECA-452 also stained about 10% of T cells in suspensions of tonsil lymphocytes (Figure 2), including both CD4+ and CD8+ T cells (data not shown). In thymocyte suspensions, only about 1% of CD2+ or CD7+ cells displayed the HECA-452 epitope (Figure 2).

Western analysis of SDS-PAGE–separated proteins indicated that the apparent M_r of the HECA-452 antigen varied among the different cell types (Figure 3). Both tonsil lymphocytes and PBL showed predominant bands at about 200 kd. These were barely detectable in lysates from the myeloid cell types. PBL also displayed a 125-kd species similar to the predominant species displayed by monocytes and the monocytoid cell line U937. However, considering that tonsil lymphocytes lacked this species, it is possible that the appearance of the 125-kd band in PBL lysates represents contamination by monocytes; in our hands, PBL preparations contain approximately 3% residual monocytes. In contrast to both monocytes and lymphocytes, PMNs showed multiple intense bands ranging from 75 kd to 160 kd, as well as a faint broad band at 55 kd to 60 kd.

The number of different specific bands identified with the HECA-452 MAb, as well as the broadness of these bands, suggests that the protein(s) identified by this MAb are heavily glycosylated. In this context, it is interesting that experiments using sodium periodate as a selective

Table 1. *Cases Studied*

Tissue/Diagnosis	Number of cases examined
Normal/Reactive Lymphoid Tissues	
Lymph node	3
Spleen	3
Gut-associated lymphoid tissues	
Tonsil	3
Appendix	2
Peyers patch	2
Thymus	3
Total	16
Inflammatory Lesions	
Gastrointestinal Tract	
Chronic gastritis/gastric lymphoid hyperplasia	1
Chronic duodenitis (nonspecific and gluten sensitivity)	2
Chronic colitis (nonspecific and Crohn's disease)	2
Lung	
Lymphoid hyperplasia (inflammatory pseudotumor)	1
Interstitial pneumonitis	2
Interstitial pneumonitis and vasculitis (rheumatoid lung)	1
Synovium	
Rheumatoid arthritis	3
Heart	
Myocarditis	1
Chronic rejection	1
Liver	
Hepatitis	2
Skin	
Allergic contact dermatitis	5
Psoriasis	3
Drug eruption	2
Lichen planus	1
Pityriasis lichenoides et varioformis acuta	1
Pityriasis rubra pilaris	1
Granuloma annulare	1
Chronic dermatitis, nonspecific	2
Other	
Lymphocytic thyroiditis	1
Chronic renal rejection	1
Nasopharyngeal lymphoid hyperplasia	1
Periorbital lymphoid hyperplasia	1
Sjogren's syndrome (minor salivary glands)	2
Total	38

oxidant for terminal carbohydrate moities of oligosaccharide side chains[32] suggest that the HECA-452 epitope itself is carbohydrate dependent. As shown in Figure 4 for U937 lysate, periodate treatment of HECA-452 antigen containing nitrocellulose strips completely abrogated the ability of HECA-452 MAb to recognize all the HECA-452–specific bands. Identical results were obtained using HECA-452 antigens from PMNs and tonsil lymphocytes (not shown). Periodate had no effect on control anti-HCAM/CD44 MAbs (Hermes-3 and H2-7), which are known to recognize carbohydrate-independent determinants.[28] Furthermore, the activity of the periodate appeared to be related to its oxidizing activity because a specific inhibitor of periodate oxidation, ethylene glycol, blocked the effect.

Characteristics and Distribution of the HECA-452+ T-cell Subset

As a first step in gaining insight into the possible functional relevance of HECA-452+ T cells, we sought to compare HECA-452 expression to markers of other functionally relevant T-cell subsets. As mentioned above, there was no correlation of HECA-452 expression with MHC class 1 or 2 restriction (ie, CD4+ or CD8+), and both TCR-$\alpha\beta$ and TCR-$\gamma\delta$ bearing T cells contained a HECA-452+ subset. Because memory T cells (or previously activated T cells) have been reported to be distinguishable from virgin T cells by their level of adhesion molecule (LFA-3/CD58, LFA-1/CD11a/18, H-CAM/Pgp-1/CD44, VLA β-chain/CD29, and CD2 molecules) and CD45R expression,[33] we investigated the possibility that HECA-452 expression may be related to previous activation by comparing expression of the HECA-452 antigen and these various markers using two-color flow cytometry of purified T cells. Memory T cells are reported to express higher levels of LFA-3, LFA-1, VLA β-chain, H-CAM, and CD2 molecules, and lower levels of the CD45R epitope than virgin T cells. MAbs TS2/9 (LFA-3) and Leu 18 (CD45R) clearly separate normal T cells into two populations (modal fluorescent intensities approximately 10 times different), whereas the modal fluorescent intensity differences between the putative memory and virgin T-cell populations for the other adhesion molecules varied from two- to four-fold, thus making the memory subset appear as a shoulder above the main peak.[33] As shown in Figure 5, essentially all the HECA-452+ T cells are found in the adhesion molecule^{high},CD45R^{low} T-cell subset, suggesting that most HECA-452 expression develops post-thymically as a consequence of activation. HECA-452 is not, however, a conventional activation antigen. Stimulation of PBMC with both PHA and Con A for 3 to 14 days did not result in an increase in the fraction of HECA-452+ T cells (illustrated in Figure 6). Indeed, in four experiments the percentage of HECA-452+ T cells at peak stimulation (day 3) was an average of 17% less than that on day 0. Furthermore, as shown in Figure 6, the mean HECA-452 fluorescent intensity was decreased after T-cell stimulation with mitogen. The decrease in T-cell HECA-452 reactivity may be due to loss of antigen by positive cells, or to preferential proliferation of the HECA-452- population. However,

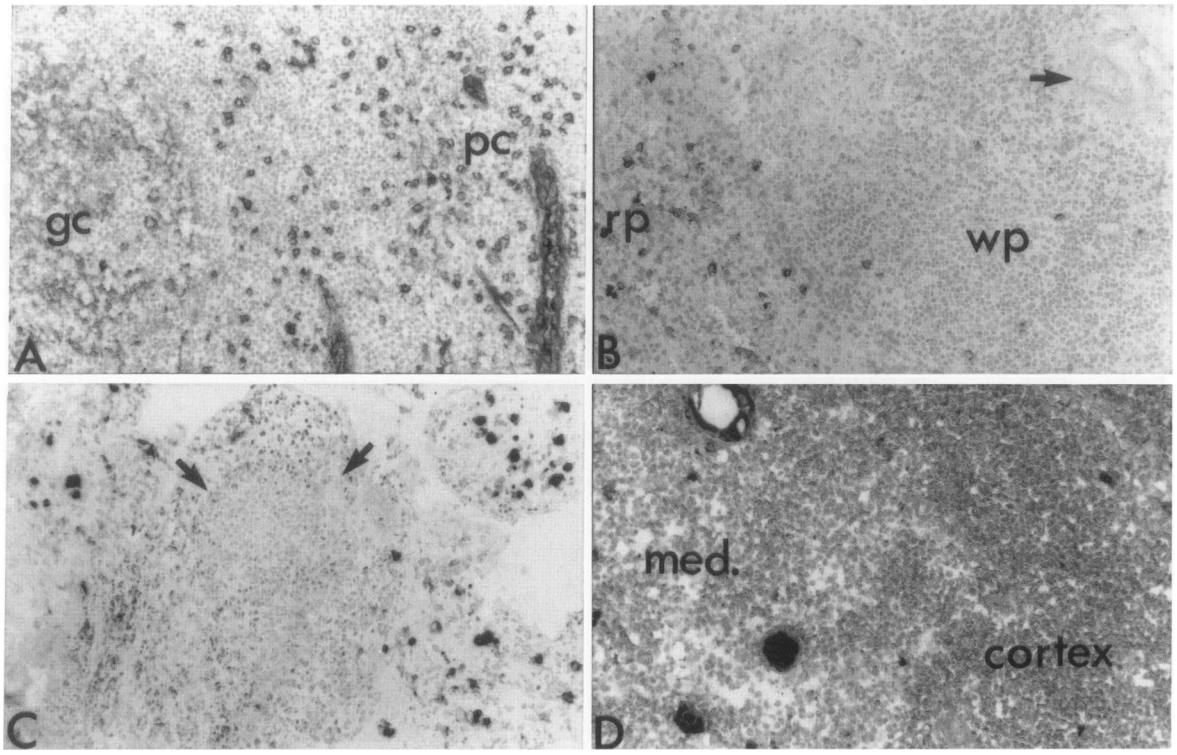

Figure 7. *Immunoperoxidase staining with HECA-452 in normal/reactive lymphoid tissues:* A: *Section of peripheral lymph node showing that HEV and about 10% of lymphocytes in the paracortex (pc) are HECA-452+. Germinal center (gc) lymphocytes are negative, but germinal center dendritic reticulum cells are weakly positive.* B: *Splenic white pulp (wp, arrow indicates penicilliary arteriole) shows only rare HECA-452 lymphocytes. HECA-452+ cells in the splenic red pulp (rp) are predominantly neutrophils.* C: *HECA-452+ cells are essentially absent in this section of ileal Peyer's patch (arrows). The positivity in the lamina propria is largely nonspecific (ie, present in control sections), although some HECA-452–expressing neutrophils are discernible.* D: *HECA-452+ thymocytes are rare but can be present in either the cortex or medulla (med.). The staining of Hassall's corpuscles in the medulla is nonspecific.*

after mitogen stimulation, HECA-452+ T cells did manifest activation antigens (ie, transferrin receptor/CD 71), suggesting that the HECA-452+ subset could respond to mitogens.

Given the observation that the HECA-452 MAb defines a subset of the putative memory T-cell population, it was of interest to determine if this subset was represented equally in diverse T-cell responses. Therefore we used tissue section immunohistology to compare the distribution of HECA-452+ T cells in 54 specimens of normal/reactive lymphoid tissues and sites of chronic inflammation (Table 1). Optimal visualization of HECA-452+ lymphoid cells required the use of frozen sections and a three-stage biotin-avidin immunoperoxidase procedure, and was critically dependent on the use of an appropriate rat IgM-specific second stage; previous studies using rat IgG-specific or mouse Ig-specific reagents failed to detect lymphoid HECA-452 reactivity.[22] HECA-452 staining patterns were similar in reactive tonsils and lymph nodes, showing staining of HEV, dendritic cells (including follicular, paracortical, and sinusoidal types), and a minor subset (10 ± 0.8%—mean ± standard error—for 6 specimens, 3 lymph node, 3 tonsil) of small lymphocytes located in the paracortical region (Figure 7a). B cells in germinal centers and mantle zones appeared to lack HECA-452 reactivity, and two-color immunofluorescence histology (HECA-452 *vs.* CD3) confirmed the T-cell nature of the paracortical HECA-452+ cells (not shown). In normal spleen (Figure 7b), peyers patches (Figure 7c) and appendix, only rare HECA-452+ lymphoid cells were present (3.5 ± 1.8% for 3 specimens, and 2.8 ± 1.3% for 4 specimens, respectively), but when observed, these cells were in T-dependent areas. Similarly, only a small minority of lamina propria or gut intraepithelial lymphocytes appeared to express the HECA-452 antigen. Polymorphonuclear leukocytes present in splenic red pulp or gastrointestinal tract mucosa were HECA-452 positive, as were PMNs in other tissue sites. In agreement with the FACS analysis mentioned above, HECA-452+ cells were also scarce in normal thymus (Figure 7d) with only about 1% to 2% positive cells scattered in both cortex and medulla. Some of these positive cells had dendritic or macro-

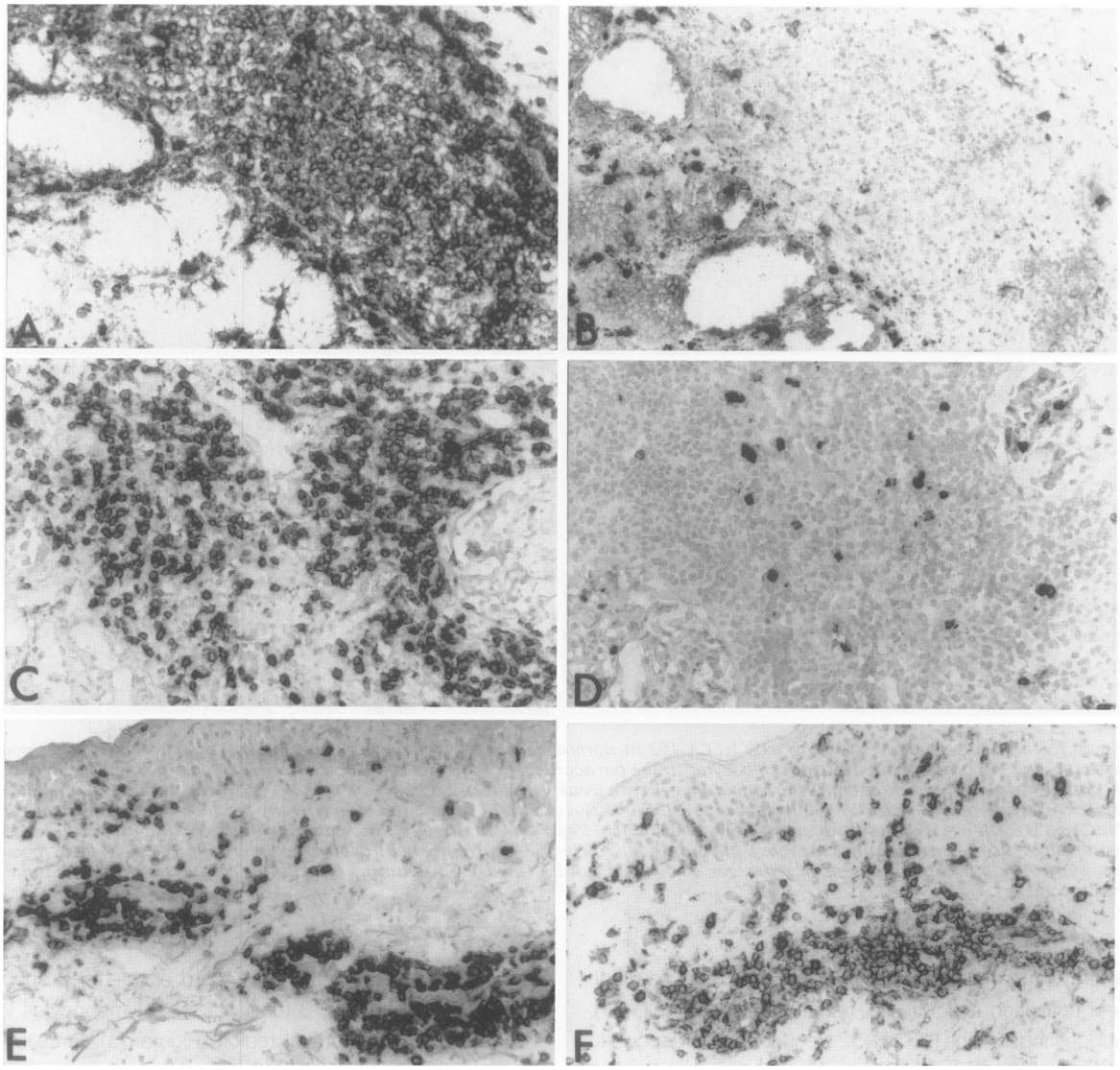

Figure 8. *Immunoperoxidase staining of chronic inflammatory infiltrate in stomach (***A, B***: chronic gastritis with lymphoid hyperplasia; mucosa in the lower left corner), kidney (***C, D***: chronic rejection, note two glomeruli in each photomicrograph), and skin (***E, F***: allergic contact dermatitis) with Leu 4/CD4 (***A, C, E***)* versus *HECA-452 (***B, D, F***). All three inflammatory sites show a predominant T-cell infiltrate, but only the cutaneous lymphoid infiltrate is significantly HECA-452+.*

phage morphology, and two-color immunofluorescence analysis of both thymic frozen sections and cytospin preparations of thymocytes indicated that less than one half of the total number of HECA-452+ cells were T lineage (data not shown).

Among 22 noncutaneous chronic inflammatory lesions, all of which had prominent, if not markedly predominant, T-cell infiltrates (including lesions of the gut, lung, synovium, liver, kidney, salivary glands, heart, thyroid, and periorbital soft tissue; Table 1), 21 had less than 10% 452+ cells within their T-cell infiltrates. Overall the noncutaneous lymphoid tissues and inflammatory sites showed a mean (±standard error) of 5% ± 1% HECA-452+ lymphocytes within T-cell areas. In striking contrast, the mean (± standard error) fraction of HECA-452+ cells within the T-cell infiltrates of 16 varied inflammatory skin lesions (Table 1) was 85 ± 2.1% ($P < 0.0005$, Students *t*-test; Figures 8 to 10). Two-color tissue section immunofluorescence analysis of five of these cases confirmed that the great majority of HECA-452+ cells coexpressed the pan T-antigen CD3 (data not shown). Although intraepidermal T cells comprise only a minor fraction of the T-cell infiltrates in these skin lesions, it is of interest to note that this population appeared to be virtually 100% HECA-452+. HECA-452 reactivity was not identified on endothelial cells within the cutaneous infiltrates studied here.

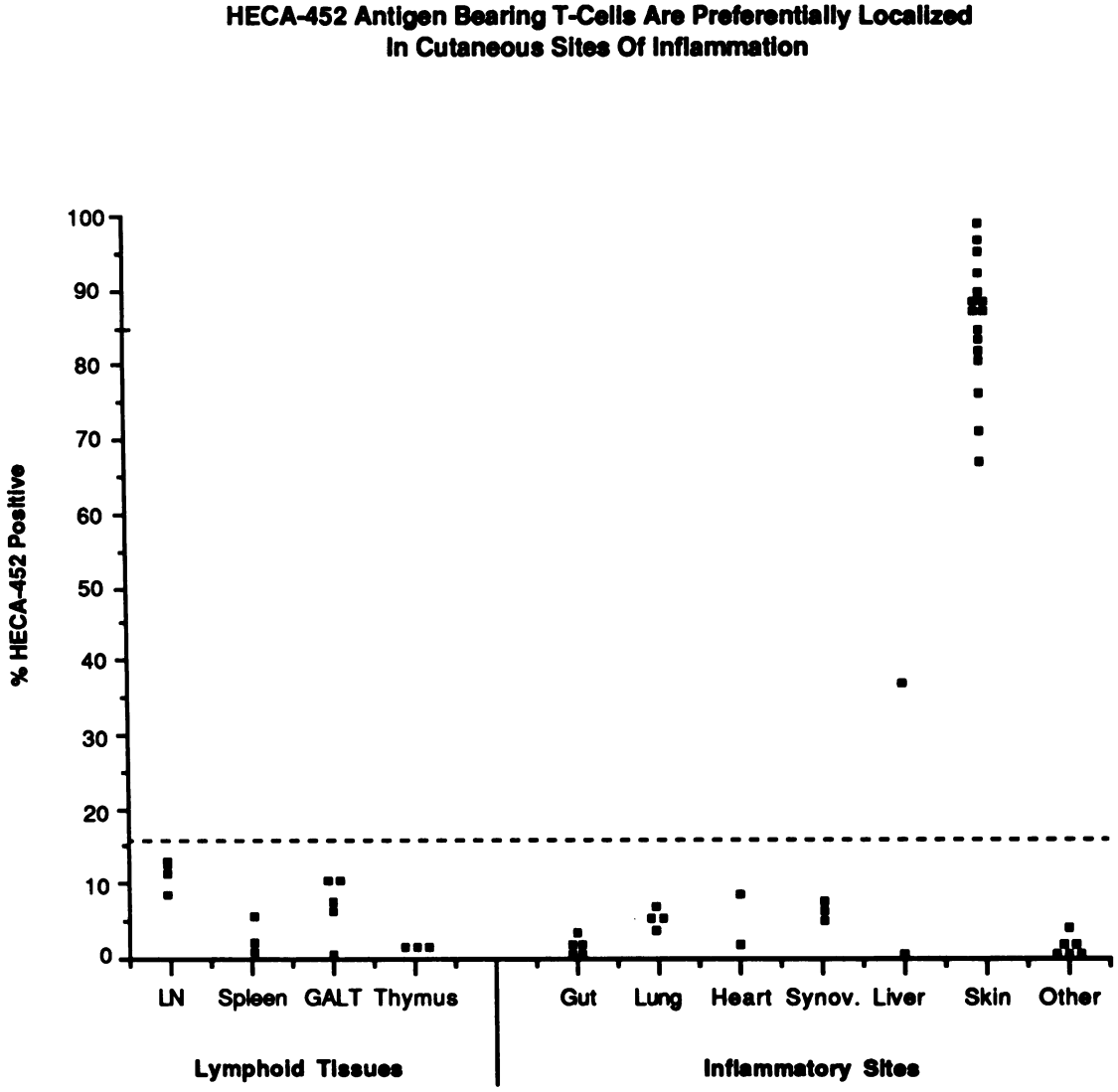

Figure 9. *Quantitative distribution of HECA-452+ lymphocytes in T-cell areas of lymphoid tissue and chronic inflammatory sites. Each data point corresponds to a case listed in Table 1. The horizontal dotted line indicates the average percentage of peripheral blood T cells that are HECA-452+.*

HECA-452 Reactivity with T-lineage Malignancies

To further explore the association between T-cell expression of HECA-452 and localization in the skin, we assessed expression of this antigen in 59 cases of T-lineage lymphoma, including 22 cases of peripheral T-lineage lymphoma, 14 cases of thymic (T lymphoblastic) lymphoma, and 23 cases of mycosis fungoides (18 of patch/plaque- or epidermotropic-stage mycosis fungoides, and 5 of tumor-stage disease). As shown in Table 2 and illustrated in Figure 11, the neoplastic cells of 16 of 18 cases of patch/plaque mycosis fungoides expressed the HECA-452 antigen. All 14 lymph node or thymic-based lymphoblastic lymphomas were HECA-452 negative, and of 22 peripheral T-lineage lymphomas, only two were HECA-452+, both of which were cutaneous lesions. Interestingly, the neoplastic cells of five cases of advanced tumor-stage mycosis fungoides, including two cases that were demonstrated to be HECA-452+ in the patch/plaque stage, were also HECA-452 negative.

Discussion

The capacity of the skin to function as an appropriate microenvironment for both the afferent and efferent arms of the immune response has been well documented; in fact, many of the classical demonstrations of both cell- and

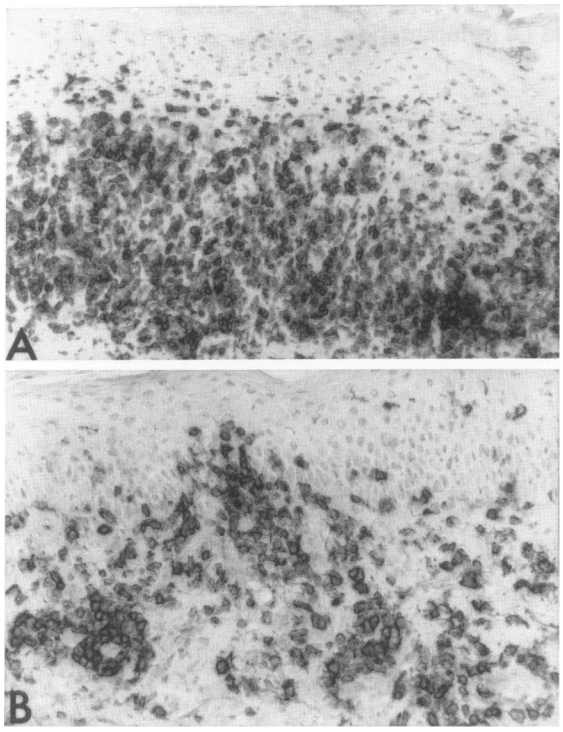

Figure 10. *Immunoperoxidase staining with HECA-452 of two additional examples of cutaneous inflammation.* **A:** *lichen planus; and* **B:** *a cutaneous drug reaction.*

antibody-mediated immune reactions have used the skin as an immunologic substrate.[13] Recent studies indicate that the skin is more than just an immunologically permissive microenvironment, but can be considered a lymphoid organ in its own right, with a characteristic architecture of lymphoid and nonlymphoid elements capable of complex interactions. This lymphoid organ, the skin-associated lymphoid tissue, has a unique organization including an epidermis capable of secreting immunoregulatory cytokines and expressing immunologically relevant adhesion/recognition molecules (eg, ICAM-1, class 2 MHC), an array of intraepidermal dendritic cells (Langerhans cells), and a complement of both dermal and epidermal T lymphocytes.[12-14,34,35]

The distinctive location of the T-cell component within SALT, particularly its intimate association with keratinocytes, implies unique functional requirements for these cells, and suggests that cutaneous T cells may represent a distinct T-cell subpopulation. This hypothesis is indirectly supported by studies suggesting that the skin may represent a novel lymphocyte homing specificity. For example, preferential lymphoid migration to skin has been shown experimentally by several investigators,[15-17] and is also clinically demonstrable in patients with the skin-localizing T-lymphoma mycosis fungoides.[18-20] Furthermore, recent studies by Sackstein et al,[36] as well as observations in our own laboratory (Wu N, Picker L, Butcher E, unpublished data), suggest that lymphocyte binding to venules in inflamed human skin is mediated, at least in part, *via* molecular mechanisms distinct from those mediating binding to lymph node or mucosal HEV. It is interesting, however, that most studies examining the immunophenotypes of T cells in both normal and inflamed skin have found that these cells resemble populations of T cells found outside the skin.[14,37,38] Similarly, analysis of large numbers of cases of mycosis fungoides indicates that while this malignancy has a characteristic immunophenotype, this phenotype is not uncommon in extracutaneous peripheral T-lineage lymphomas.[30,37,38]

Here we report that cutaneous T cells, both benign and malignant, differ from their counterparts in noncutaneous sites by their expression of an epitope recognized by the MAb HECA-452. This MAb, which recognizes a 200-kd molecule on lymphocytes, reacts with the great majority of T cells in cutaneous inflammatory sites and the cells of most skin-based T-cell lymphomas, but with only a small fraction of T cells in a wide variety of noncutaneous lymphoid tissues and inflammatory sites, and not at all with non-skin–based T-cell malignancies. The observations that very few thymocytes express the HECA-452 epitope, and that the 15% or so peripheral blood T cells that express this epitope belong to the putative memory T-cell subset (the adhesion molecule^{high}, CD45R^{low} population[33]) suggest that lymphocyte expression of the HECA-452 epitope may develop peripherally as a consequence of antigenic stimulation. Because nonspecific stimulation of peripheral blood T cells by mitogens does not increase HECA-452 epitope expression, it is likely that special conditions are required for the induction of this determinant.

Table 2. *HECA-452 Antigen Expression in T-lineage Malignancies*

	No. of HECA-452+/total
Peripheral T-lineage Lymphoma*	
Skin	2/7
Lymph node	0/13
Spleen	0/1
Lung	0/1
Total	2/22 (9%)
Thymic (T-lymphoblastic) Lymphoma	
Lymph node/thymus	0/14 (0%)
Mycosis Fungoides	
Patch/plaque stage	16/18 (89%)
Tumor stage	0/5
Total	16/23 (70%)

* The peripheral T-lineage tumors included 9 mixed or angioimmunoblastic-like lymphomas, 11 large-cell or immunoblastic lymphomas (including the 2 HECA-452+ cases), and 2 monomorphic medium-sized lymphomas.

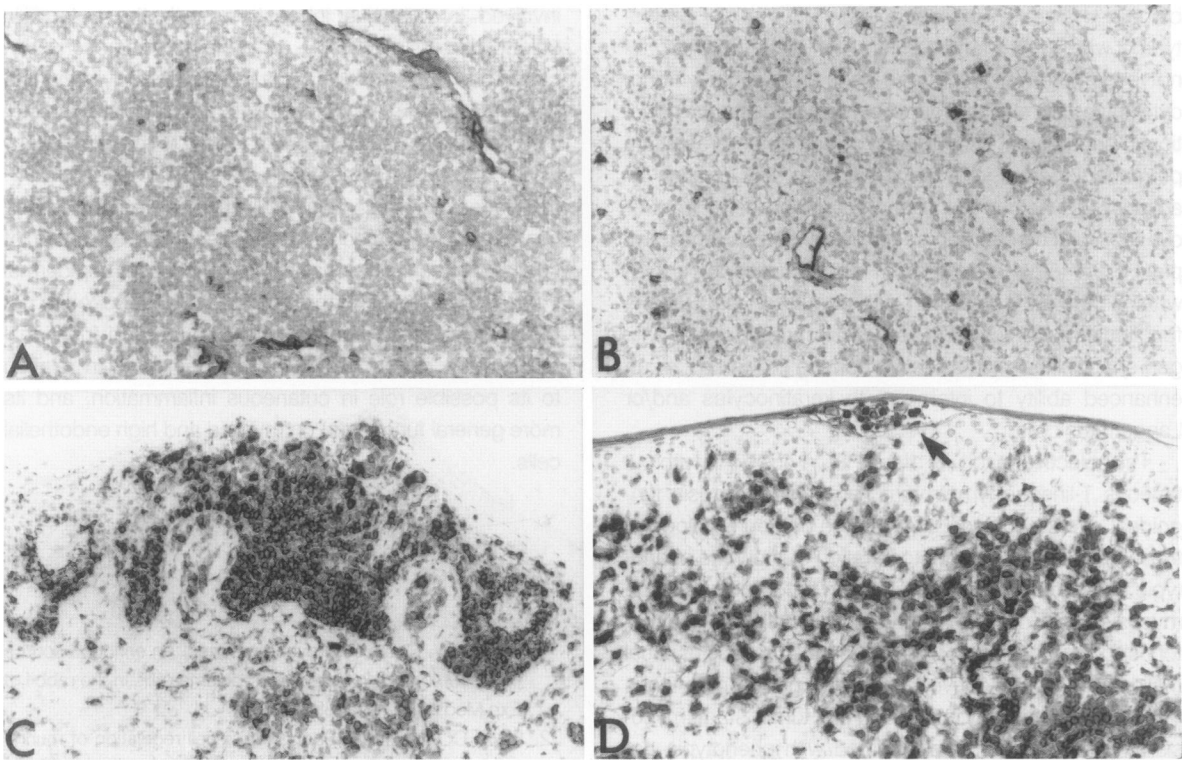

Figure 11. *Immunoperoxidase staining with HECA-452 of T-lineage lymphoma: HECA-452-negative T-lymphoblastic (thymic) lymphoma* (A), *and peripheral T-cell lymphoma* (B), *both in lymph node (note that residual HEV and occasional reactive cells are positive)* versus *HECA-452+ patch/plaque stage mycosis fungoides* (C, D). *Arrow in* D *indicates Pautrier's microabscess with positive tumor cells.*

A simple model consistent with these findings is that a portion of recent thymic emigrants and virgin T cells—initially HECA-452 negative, and non-tissue-selective in homing behavior[5]—are first stimulated in the context of an immune reaction at a cutaneous site. In this location, perhaps as a consequence of interaction with keratinocytes, specific induction of the HECA-452 epitope will occur. This induction can occur in both MHC class 1- (CD8+) and class 2- (CD4+) restricted T cells, as well as in T cells expressing the $\gamma\delta$-T-cell receptor. It may also occur less frequently in B lymphocytes. Along with HECA-452 antigen induction, this activation would result in up-regulation of multiple general cell adhesion molecules characterizing the memory phenotype of HECA-452+ cells. It is also possible that associated with the HECA-452 induction (or perhaps as a consequence of it), the homing properties of these lymphocytes will change such that the efficiency of skin localization is increased (see below). The result of this process would be the observed phenomena that nearly all skin-associated T lymphocytes would express the HECA-452 epitope (either by specific local induction or preferential migration), whereas at other sites only those few HECA-452+ lymphocytes nonspecifically passing through would be present. According to this model, low-grade malignant transformation of these cutaneous T cells might be expected to result in a HECA-452+ skin-localizing lymphoma, which is exactly what was observed in our series of patch/plaque-stage mycosis fungoides.

The preferential expression of the HECA-452 epitope on cutaneous T-lymphocyte populations is, to our knowledge, the first clear demonstration of phenotypic differences between skin-associated and non-skin–associated lymphocytes. However, precedent for this finding has been set in the gut, where the molecules recognized by the HML-1 MAb in humans,[3] and the RGL-1 MAb in the rat[4] have been shown to be preferentially expressed on gut-associated lymphocytes. The HML-1 MAb has also been shown to react selectively with enteropathy-associated T lymphomas.[39] These phenotypic data suggest the existence of tissue-specific lymphocyte subpopulations, and, together with the data demonstrating that certain lymphoid populations (particularly previously activated lymphocytes, either blasts or small memory cells) exhibit tissue-specific homing behavior,[5–10,15–17,20] support the hypothesis that the immune system may be composed of a series of parallel, tissue-specific circuits in which memory lymphocyte populations adapted or selected to function optimally in a particular microenvironment continuously recirculate to the same microenvironment *via* the

blood and lymph. With respect to the HECA-452+ phenotype, it will be important to determine 1) whether this phenotype is potentially reversible once a particular lymphocyte leaves the skin microenvironment (requiring reinduction in subsequent skin lesions) or perhaps represents a permanent indication of commitment to skin immune reactions; 2) whether this phenotype is exclusive from or overlaps with other organ-specific phenotypes (for example, HECA-452+ *vs.* HML-1+ T-cell populations); and 3) whether the HECA-452+ T-cell population has unique functional properties such as a distinct repertoire of antigen specificities, skin-selective homing properties, or an enhanced ability to interact with keratinocytes and/or Langerhans cells.

The selective reactivity of the HECA-452 MAb with cutaneous T-lineage lymphomas, especially mycosis fungoides, offers additional clues to the pathophysiology of these neoplasms. The demonstration of a unique phenotypic characteristic common to both patch/plaque-stage mycosis fungoides and the reactive T cells in inflammatory skin lesions provides new evidence that this unusual malignancy is likely derived from these normal cutaneous T cells. It is particularly interesting that tumor-stage mycosis fungoides, which is associated with phenotypic dedifferentiation (ie, surface antigen loss), loss of epidermotropism, and widespread dissemination,[18,19,30,38] lacked HECA-452 expression in our study (including cases known to be HECA-452+ in previous biopsies with patch/plaque-stage disease). This intriguing correlation not only supports a role for the HECA-452 epitope in the mechanism of skin-localization (see below) but also has potential clinical-diagnostic significance: loss of tumor cell HECA-452 reactivity might precede clinical progression of patch/plaque-stage disease, and therefore may be a useful predictor of those mycosis fungoides patients at high risk for transition to a more aggressive stage of their disease.

The function of the molecule(s) bearing the HECA-452 epitope can only be speculated on at this time. The observations that this epitope is present on PMNs, monocytes, a subset of lymphocytes, as well as on HEV, is present on molecules of different M_r, and appears carbohydrate dependent suggest the possibility that this epitope may define a novel post-translational modification common to a number of glycoproteins, possibly on adhesion molecules analogous to the L2 and L3 family of glycoproteins in the nervous system. The L2 and L3 determinants are carbohydrate dependent and are expressed on distinct polypeptides that have in common a cell adhesion/recognition function in neural development.[40,41] Among lymphocytes, the preferential expression of the HECA-452 epitope by skin-associated T cells suggests that the lymphoid molecule(s) bearing this determinant may be involved in adhesive interactions with the potentially unique aspects of SALT — for example, interactions with cutaneous endothelium (ie, a skin-specific lymphocyte homing receptor), or perhaps interactions with keratinocytes or Langerhans cells. With regard to the latter possibilities, the recent observation that otherwise identical T-cell clones can differ in their ability to localize in the epidermis after dermal inoculation suggests the operation of specific molecular mechanisms promoting intraepidermal T-cell localization.[42] Further studies will be required to define more precisely the molecular nature and functional significance of the HECA-452 epitope, both in relation to its possible role in cutaneous inflammation, and its more general function(s) on myeloid and high endothelial cells.

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Acknowledgments

The authors thank Dr. R. Warnke, G. Wood, S. Jalkanen, and N. Wu for their assistance in obtaining the clinical material used in this study, P. Verzola for photographic assistence, and Drs. E. Berg, M. Jutila, R. Hallman, K. Kishimoto, and R. Bargatze for critical review of the manuscript.